

DEVELOPMENT OF AN IMMUNOENZYMATIC ASSAY USING A MONOCLONAL ANTIBODY AGAINST A 50-kDa CATABOLITE FROM THE P126 *PLASMODIUM FALCIPARUM* PROTEIN TO THE DIAGNOSIS OF MALARIA INFECTION

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The WHO criterion of deferring any donation of blood by a confirmed case of malaria for three years after cessation of therapy can not be applied in areas where malaria is endemic. For this reason we developed an immunoenzymatic assay for the detection of plasmodial antigens for blood screening in malarial endemic areas. So, we tested sera from 191 individuals. Among patients with active disease 100% of the cases of Plasmodium falciparum or mixed infections and 91.7% of those with P. vivax were positive for the presence of plasmodial antigens. The lower parasitaemia detected was 0.0003% for P. falciparum and 0.001% for P. vivax malaria. When the frequency of positive circulating malarial antigens was evaluated among asymptomatic and symptomatic individuals with negative TBS, positive results were found in respectively 38.7% and 17.7% of the individuals studied in the 30 days after confirmed malaria attack. Data provide by these assays have shown that ELISA seemed to be more sensitive than parasitological examination for malaria diagnosis. This test by virtue of its high sensitivity and the facilities in processing a large number of specimens, can prove to be useful in endemic areas for the recognition of asymptomatic malaria and screening of blood donors.

Key words: *Plasmodium falciparum* – *Plasmodium vivax* – diagnosis – plasmodial antigen – monoclonal antibody – immunoenzymatic assay

It is well known that in urban areas of endemic regions transmission of malaria infection represents a risk for transfusion medicine. In countries where the disease is not prevalent, the facility of travel from and to endemic countries, has also increased the chances of including infected individuals among asymptomatic donors. The WHO criterion of deferring any donation of blood by a confirmed case of malaria for three years after cessation of therapy can not be applied in areas where malaria is endemic. Donor screening by blood smear examination is labour intensive and not sensitive enough for use on a large scale (Mackey et al., 1980). Conversely malarial antibody detection is of no value in blood banks located in areas

where malaria is endemic since the presence of antiplasmodial antibody, very frequent in these regions, does not necessarily reflect active infection.

The detection of *Plasmodium falciparum* circulating antigens has been proposed by several authors as a strategy to diagnose malaria infection (Mackey et al., 1982; Avraham et al., 1982; Londer et al., 1987; Khusmith, 1988; Kusmith et al., 1988; Dubarry et al., 1990) and recently it was demonstrated that this method could be more sensitive than microscopic examination (Kusmith et al., 1988). Data provided by such a sensitive test could be used as an indicator of present or recent infection for blood screening in malarial endemic areas.

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With the availability of an appropriate monoclonal antibody (Delplace et al., 1985), we have standardized an ELISA test to search for plasmodial circulating antigens. The original procedure (Fortier et al., 1987) was modi-

fied and is now 10 times more sensitive. This increase in sensitivity enabled us a) to confirm malaria diagnosis in *P. falciparum* and *P. vivax* parasitized individuals; b) to detect malaria infection in symptomatic individuals with negative thick blood smear and c) to detect plasmodial antigens in asymptomatic subjects with negative thick blood smear and recent history of malaria.

MATERIALS AND METHODS

Serum samples – Sera were obtained from subjects living in endemic region (Ariquemes - Rondônia) of the Brazilian Amazon. They corresponded to 174 polyinfected individuals and 17 cases of primo-infection. These individuals were distributed in three groups: group A consisted of 70 malarious patients with positive thick blood smear (TBS), 34 with *P. falciparum*, 33 with *P. vivax* and 3 with mixed infections; group B consisted of 25 symptomatic individuals with negative TBS and studied at different intervals after the last parasitologically confirmed attack of malaria (due to *P. falciparum* in 16 cases, to *P. vivax* in 8 and to a mixed infection in 1 case) and; group C comprised 96 asymptomatic individuals studied at different intervals after a last positive TBS (61 *P. falciparum*, 33 with *P. vivax* and 2 with mixed infections).

Determination of species plasmodium – The diagnosis of malaria was performed by microscopic examination of 500 fields of Giemsa-stained thick or thin blood smears. The parasitaemia was assessed by examination of at least 200 leucocytes in thick blood film.

Hyperimmune polyclonal antibodies – A pool of sera with malaria fluorescent antibody titers ranging from 640 to 1280 was obtained from individuals with a past history of malaria and negative TBS and used as a source of polyclonal antibodies. All sera were obtained from individuals living in the same endemic area (Ariquemes - Rondônia). Two preparations were made: one (pool A) constituted predominantly by immunoglobulins from individuals with a past history of *P. vivax* malaria or of mixed infection, and the other (pool B) comprised immunoglobulins from *P. falciparum* infected subjects. The procedure used for purification of the globulin fraction was salt precipitation. The titer of anti-malarial antibody for both anti-plasmodium Ig preparations was 5120.

Monoclonal antibodies – A monoclonal, mouse IgG1 antibody (Mab 23D5), specific to an epitope of a 50 kDa exoantigen (P50), which is a breakdown product of a 126 kDa schizont protein, the major component of supernatant collected at the time of schizont rupture (Delplace et al., 1985) was used as a probe for the detection of *P. falciparum* antigens.

Antigens and peptide – Three *P. falciparum* antigens were tested: a) the 245 antigen which corresponds to a protein of 100 kDa present in blood merozoite (a kind gift from Dr Pierre Bayard, Unité Inserm U 313, Paris-France), b) a 1:100 dilution of a supernatant of distilled water (v/v) lysed parasitized red blood cells from "in vitro" *P. falciparum* culture and c) a crude antigen obtained from asynchronous culture by "the candle jar" method (Trager & Jansen, 1976) prepared as follows: Parasitized red blood cells were washed (350 x g - 10 min - 4 °C) three times in RPMI medium (GIBCO) and lysed by the addition of saponin to 1% final concentration. The pellet was resuspended in carbonate buffer 0.1 M, pH 9.6, sonicated three times (5 min each) on ice and centrifuged at 7000 g for 15min at 4 °C. Protein concentration in the supernatant was estimated according to Johnstone & Thorpe (1988). A *P. falciparum* peptide (307) specific to hepatic schizont was also assayed (Druilhe et al., 1984).

Immunofluorescent antibody test – *P. falciparum* blood forms (isolate 2486 from a patient from PiriQUITOS, Rondônia, Brazil), were grown *in vitro* in human erythrocytes according to established techniques (Trager & Jensen, 1976). Sera were assayed for anti-blood stage antibodies according to the standard immunofluorescence test (Sulzer et al., 1969). For the preparation of antigen slides we used the formula proposed by Ferreira & Sanchez (1988) to obtain a mean of 20 infected red blood cells per field with 40xs microscope objective. Sera were tested in a two-fold serial dilution for antiplasmodial IgG and IgM antibodies. A reciprocal titer of 20 or greater was considered positive.

Four-step ELISA for antigen detection – Wells of microtiter plates (Nunc, Denmark) were incubated overnight at 4 °C with 500 ng per well of monoclonal antibody 23D5 diluted in PBS. The wells were washed three times with PBS-Tween 20 0.05% (PBS-T20) and incubated overnight with 150 µl of carbonate buffer 0.06M pH 9.6, containing 5% of skim

milk. Test serum, diluted at the same volume in PBS-Tween 20 0.05% plus 1% of skim milk (PBS-T20-SM) was heated in a water bath at 56 °C for 15 min and centrifuged during 5 min in a microfuge (Beckman). The test serum-supernatant was then incubated during 2 h at 37 °C and assayed in duplicate. To assess the binding of *P. falciparum* antigens to the monoclonal coated plate, 100 µl of a 1:40 diluted polyclonal hyperimmune human gamma globulin in PBS-T20-SM was added to each well. Plates were incubated for 1 h at 37°C and washed three times with PBS-T20. Then 100 µl of a goat anti-human IgG labeled with peroxidase diluted 1:1000 (Sigma) in PBS-T20-SM was added to each well. After incubation for 1 h at 37 °C and three washes with PBS-T20, 200 µl of substrate solution (0.4 mg/well o-phenylene-diamine in citrate-phosphate buffer pH 5.0) was added and the reaction stopped by addition of H₂SO₄ 2N. Optical density (OD) was measured at 492nm. The average OD of normal human sera with no past history of malaria + 2 standard deviations was defined as the upper limit of the normal range.

RESULTS

Detection of antigens with different ELISA P. falciparum — Protocols — The sensitivity of four different protocols in relation to *P. falciparum* antigens was assessed: a) by a three-step ELISA, using antigen coated plates; b) by a four-step ELISA using the same monoclonal 23D5 or human polyclonal antibody as coating and second antibodies or c) using Mab 23D5 as coating and polyclonal as second antibody or vice versa. The monoclonal-polyclonal sys-

tem was shown to be the most sensitive (Table I) and consequently this protocol was selected for the detection of circulating malarial antigens in human sera.

Both preparations of polyclonal antibodies were used and the results in a qualitative sense were always the same independently of the preparation used. The differences observed concerned only the OD values, which were lower for detecting *P. vivax* malaria when the pool of human sera was made only with polyclonal *P. falciparum* antibodies.

Detection of malarial antigens in human sera — Among patients with active disease (Group A) 100% of the cases of *P. falciparum* or mixed infections and 91.7% of those with *P. vivax* malaria were positive for the presence of plasmodial antigens. The lower parasitaemia detected was 0.0003% for *P. falciparum* and 0.001% for *P. vivax* malaria (Table II). No direct correlation was observed between parasitaemia and ELISA ratio values.

When the frequency of positive circulating malarial antigens was evaluated among symptomatic individuals (Group B) tested after the last parasitologically confirmed attack of malaria studied less than 30 days, between 30 and 180 days and between 180 and 360 days, positive results were found in 33.3%, 66.6% and 20% individuals, respectively (Table II). Regarding asymptomatic individuals (Group C) with negative TBS, studied also at the same interval periods, positive results were found in 38.7% of the individuals studied in the 30 days after the confirmed malaria attack. No positive

TABLE I

Comparison of the ability of monoclonal (Mab) and polyclonal (Pab) antibodies in recognizing and binding *Plasmodium falciparum* antigens in protocols using antigens (Ag) or antibodies as coating material

Antigens	ELISA Protocols					
	Ag coated plate			Antibody coated plate		
	Mab ^a	Pab ^a	Mab ^a /Mab ^c	Pab ^b /Pab ^c	Mab ^b /Pab ^c	Pab ^b /Mab ^c
Lysed pRBC	—	+	bl	+	+	+
Crude Ag	+	+	—	—	+	bl
Ag 245	+	+	—	—	+	bl
PT 307	—	—	—	—	—	bl

a: single antibody; b: coating antibody; c: second antibody; pRBC; lysed parasitized red blood cells; bl: border, line; Ag 245 — 100kDa blood merozoite; PT 307 — peptide specific to hepatic shizont.

TABLE II

Frequency of individuals with plasmodial circulating antigens among malarious patients and symptomatic or asymptomatic individuals at different intervals after a last parasitologically confirmed attack of malaria

Days after last Positive TBS	Symptomatic individuals		Asymptomatic individuals	
	Positive ELISA NP/N (%)	X ± SD (days)*	Positive ELISA NP/N (%)	X ± SD (days)*
Positive TBS	Pf 37/37(100.0)(a, 1) Pv 33/36(91.7)(a, 2)	—	—	—
≤ 30	Pf 2/6 (33.3) Pv 1/3 (33.3)	13.8 ± 9.4 18.6 ± 11.0	Pf 9/20(45.0)(c) Pv 4/12(33.0)(c)	12.2 ± 5.8 12.2 ± 8.0
≤ 30 ≤ 180	Pf 6/9 (66.6) Pv 3/5 (60.0)	96.6 ± 29.1 92.0 ± 44.3	Pf 0/22 (0.0)(b) Pv 0/13 (0.0)(b)	130.9 ± 27.0 115.6 ± 33.7
≤ 180 ≤ 360	Pf 1/2 (50.0)(b) Pv (0/1 (0.0)(b)	255.0 ± 21.2 255.0 ± 21.2	Pf 4/21 (19.0) Pv 1/10(10.0)	215.7 ± 39.8 216.0 ± 56.2

TBS: thick blood smear; Pf: *Plasmodium falciparum*; Pv: *P. vivax*; NP: number of positive individuals, including positive results in (a) 3 out of 3, (b) 0 out of 1 and (c) 1 out of 1 individuals with mixed (Pf + PV) infections; *mean time elapsed after last positive TBS. Parasitaemia ranging from (1) 0.0003% to 0.9% and (2) 0.001% to 0.5%.

result was recorded between 30 and 180 days after positive TBS although 16.1% of individuals tested >180<360 days carried circulating antigens (Table II).

Comparison of antigen detection and direct parasitological examination — We next studied the sensitivity of the antigen detection assay comparatively to that of the parasitological examination. ELISA seemed to be more sensitive than TBS for malaria diagnosis since 3 out of 9 symptomatic individuals and 12 out of 31 asymptomatic subjects, examined during the 30 day-period after the parasitological confirmation of malaria, were found to carry antigens even in the absence of detectable parasites at the direct examination (Table II).

Comparative detection of antigens and antibodies — Comparing the detection of antigens by ELISA and of antibodies by IFAT, in prime and polyinfected patients with parasitologically confirmed malaria, we observed that for both groups of individuals antigen screening was more sensitive than the antibody detection. The sensitivity for antigen detection seems to be greater for prime than for polyinfected individuals, however actually all primeinfected individuals studied have had *P. falciparum* malaria, and the negative results obtained were those related to *P. vivax* malaria (Fig.).

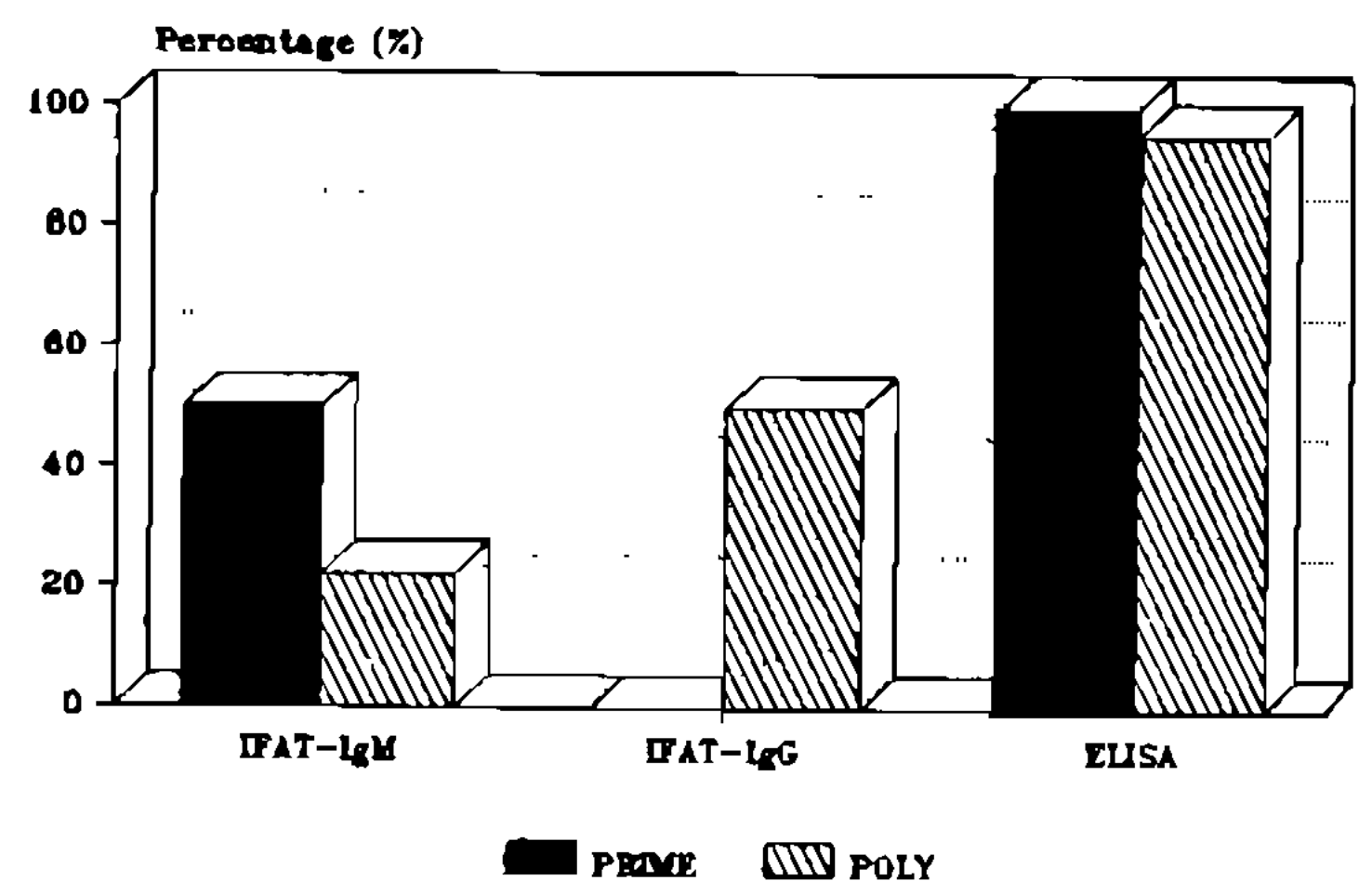


Fig. 1: comparison of antigen (ELISA) and antibody (IFAT) detecting methods in prime and polyinfected malarial patients with positive thick blood smear (TBS).

DISCUSSION

The prime purpose of an immunological procedure aiming at the detection of plasmodial antigens would be to identify people with low levels of parasitaemia not detected by microscopic examination. Our assay was shown to be very sensitive since it was capable of detecting circulating plasmodial antigens not only in 100% of patients with parasitologically confirmed *P. falciparum* malaria but also in 33.3% of symptomatic individuals with negative TBS and even in 38.7% of asymptomatic subjects examined up to 30 days after the last positive TBS.

The higher sensitivity of the test, as compared to that observed with the original proce-

ture (Fortier et al., 1987), may be due to: a) the concentration of the monoclonal antibody utilized for coating plates (about twice that used by Fortier et al., 1987) and or b) the fact that we have used a gamma globulin fraction from a pool of hyperimmune sera instead of a whole human sera as a source of polyclonal antibodies. One other fact that merits to be emphasized is the ability of the present assay to detect plasmodial antigens in *P. vivax* infected individuals. This property, absent in the original test, is probably due to the use of a pool of sera containing also anti-*P. vivax* antibodies as suggested by the higher OD values observed in *P. vivax* sera when pool A (rich in anti-*P. vivax* antibodies) was used. This observation demonstrated the existence of *P. falciparum* - *P. vivax* common epitopes in the 50-kDa protein.

No linear correlation between parasitaemia and ELISA ratio values was observed. This fact, already observed by other investigators (Taylor et al. 1986; Fortier et al., 1987) could be due to the longer clearance of soluble antigens than of cellular ones (Dubarry et al., 1990).

It has been claimed that the immunodiagnosis test based on human sera as the source of antibodies makes the standardization of the assay practically impossible due to the heterogeneity in antibody content and consequently the use of monoclonal antibodies instead of polyclonal ones has been suggested (Khusmith, 1988). Based on this presumption we tested the sensitivity of the ELISA in four different protocols and the one that showed the best results was that using the monoclonal-polyclonal system. This was probably due to the fact that specific and non specific polyclonal antibodies compete during the plate coating procedure, impairing the sensitivity of the assay if polyclonal antibodies are used to coat the plate. Conversely, polyclonal antibodies used as second antibody can bind to epitopes other than that recognized by the monoclonal antibodies.

In our study an increase in the frequency of antigen detection was observed with time after the last parasitologically confirmed malaria. This fact in our opinion, may reflect the increased risk of reinfection in studied individuals that were still living in endemic areas.

Comparison of antigen detection by ELISA and antibody screening by IFAT, in parasitized (positive TBS) individuals, point to a greater

sensitivity of the former, suggesting that the presence of circulating antigens can be a better indicator of disease activity. The demonstration of serum antibodies by IFAT was however found to be more frequent when prime and polyinfected (negative TBS) individuals were studied at different times after a parasitologically confirmed attack of malaria. Obviously, since anti-plasmodial antibodies can remain for months after disappearance of the plasmodium from the organism (and therefore their presence cannot be directly correlated with a present or recent episode of malaria infection), the observation of antigen negative-antibody positive individuals can hardly be interpreted as an evidence of a greater sensitivity of the antibody detection approach. The main question that remains however to be answered is to know whether or not these subjects are actually parasite free individuals, whose blood can be used safely for donation purposes.

In the same way the present study showed that between 30 and 180 days after parasitological cure, the individuals are free of circulating antigens and further studies are necessary to determine whether the donation of antigen free blood in this period is safe.

Finally the assay, by virtue of its sensitivity and ease in processing a large number of specimens, can be useful in endemic areas for the recognition of asymptomatic malaria and the screening of blood donors.

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